

EFFECTS OF CONTINUOUS ADDITION OF NITRATE TO A THERMOPHILIC  
ANAEROBIC DIGESTION SYSTEM

BY

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# TABLE OF CONTENTS

|                                                                                                                     | Page |
|---------------------------------------------------------------------------------------------------------------------|------|
| ACKNOWLEDGMENTS . . . . .                                                                                           | ii   |
| ABSTRACT . . . . .                                                                                                  | v    |
| INTRODUCTION . . . . .                                                                                              | 1    |
| MATERIALS AND METHODS . . . . .                                                                                     | 6    |
| Digester Construction and Operation . . . . .                                                                       | 6    |
| Gas Analysis . . . . .                                                                                              | 7    |
| pH, Volatile Fatty Acid, and Ion Analysis . . . . .                                                                 | 8    |
| Stable Isotope ( <sup>15</sup> N-Nitrate) Experiments . . . . .                                                     | 10   |
| Digester Effluent Composition and Particle Size Distribution Analysis . . . . .                                     | 11   |
| Enumerations of Digester Microflora . . . . .                                                                       | 12   |
| RESULTS . . . . .                                                                                                   | 15   |
| Nitrate Introduction to the Thermophilic Anaerobic Digestion System . . . . .                                       | 15   |
| Analysis of Digester Gas Production and Composition, Volatile Fatty Acids, Ammonium, and pH After Feeding . . . . . | 16   |
| Gas Production . . . . .                                                                                            | 16   |
| Gas Composition . . . . .                                                                                           | 18   |
| Volatile Fatty Acids in the Sludge . . . . .                                                                        | 18   |
| Ammonium Ion Pools in the Sludge . . . . .                                                                          | 19   |
| Sludge pH Changes . . . . .                                                                                         | 19   |
| Inhibition Resulting from Nitrate, Nitrite, and Ammonium Addition . . . . .                                         | 19   |
| Nitrate Reduction Kinetics . . . . .                                                                                | 20   |
| Nitrite Reduction Kinetics . . . . .                                                                                | 21   |
| Nitrous Oxide Reduction . . . . .                                                                                   | 22   |
| Stable Isotope Experiments . . . . .                                                                                | 23   |
| Effluent Analysis and Particle Size Distribution . . . . .                                                          | 23   |
| Enumerations of Microflora in Digester Sludge . . . . .                                                             | 23   |
| DISCUSSION . . . . .                                                                                                | 75   |
| Nitrate Introduction . . . . .                                                                                      | 75   |
| Gas Production and Composition . . . . .                                                                            | 76   |
| Changes in Digester Parameters Measured . . . . .                                                                   | 77   |
| Fatty Acid Pools . . . . .                                                                                          | 77   |
| Ammonium Pools . . . . .                                                                                            | 77   |
| Sludge pH . . . . .                                                                                                 | 77   |

|                                                                                                 | Page |
|-------------------------------------------------------------------------------------------------|------|
| Analysis of Digesters Pumped Calcium Nitrate, Sodium Nitrate,<br>or Ammonium Chloride . . . . . | 78   |
| Inhibitory Effects of Nitrate, Nitrite, and Ammonium . . .                                      | 78   |
| Reduction of Nitrate and Nitrite . . . . .                                                      | 79   |
| Nitrous Oxide Reduction . . . . .                                                               | 80   |
| Stable Isotope Experiments . . . . .                                                            | 81   |
| Analysis of Effluent Composition, Particle Size Distribution,<br>and Microflora . . . . .       | 81   |
| Effluent Composition . . . . .                                                                  | 81   |
| Effluent Particle Size Distribution . . . . .                                                   | 82   |
| Enumerations of Sludge Microflora . . . . .                                                     | 82   |
| CONCLUSION . . . . .                                                                            | 85   |
| REFERENCES . . . . .                                                                            | 87   |
| BIOGRAPHICAL SKETCH . . . . .                                                                   | 91   |

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EFFECTS OF CONTINUOUS ADDITION OF NITRATE TO A THERMOPHILIC  
ANAEROBIC DIGESTION SYSTEM

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The biodegradation of complex organic matter is regulated partially by the ability to dump electrons which build up in the form of reduced nicotinamide adenine dinucleotide (NAD). The addition of oxidants to lower levels of NADH, or hydrogen, produced upon NADH oxidation, may serve to increase the rate and extent to which complex organic matter is degraded.

The effects of the continuous addition of the oxidant, nitrate, were investigated on a single-stage, thermophilic, anaerobic digester. The digester had a sludge/liquid volume of 4 liters and was mixed semi-continuously. The digester was maintained at 55°C and batch fed every 24 hours with a detention time of 20 days. The digester acclimated rapidly to nitrate addition and was maintained at a pump rate of 0.4  $\mu\text{moles NO}_3/\text{ml sludge/hour}$ . The continuous addition of nitrate resulted in a constant inhibition of total gas (30%) and methane production (36%). Reduction in total gas and methane production was accompanied by

increases in sludge pH and acetate, propionate, and ammonium ion pools. Compared to the control digester sludge, the nitrate-pumped sludge was higher in percentages of dry matter and volatile solids while lower in percentages of acid detergent fiber and cellulose plus cutin. Effluent particle size distribution revealed a shift to smaller particle sizes in the nitrate-pumped sludge. The continuous addition of nitrate resulted in lower numbers of methanogens and sulfate reducers in the sludge, with increases in nitrate-reducing and cellulose-degrading microorganisms. These findings indicate that added nitrate underwent dissimilatory reduction to ammonium ion, as determined from gas analysis, ammonium pools, and  $^{15}\text{N}$ -nitrate-label experiments.

Continuous nitrate addition to a single-phase digestion system was determined to inhibit methane production from biomass and wastes. Thus for the single-stage digestion system in which maximum methane production is desired, the addition of nitrate is not recommended. However, in a multistage digestion system, the continuous addition of nitrate in the primary stage to increase the rate and extent of degradation of organic matter to volatile fatty acids, which then would serve as feed to a second stage, may be advantageous.

## INTRODUCTION

In the search for renewable sources of energy, methane generation from biomass and wastes has gained increased attention.

The degradation of complex organic matter to methane results in relatively clean and easily purified gaseous products, carbon dioxide and methane, with a relatively small growth yield of bacteria (30,31). Thus a large amount of organic matter is destroyed but about 90% or more of the substrate energy is retained in the methane produced. The degradation of complex organic matter is thought of in three stages. The first stage involves species of fermentative bacteria which as a complex metabolic group hydrolyze polysaccharides such as cellulose and degrade the products of these to organic acids, hydrogen, and carbon dioxide. The second stage bacteria, collectively called the hydrogen-producing acetogenic bacteria, obtain energy for growth by producing acetate and hydrogen, and sometimes carbon dioxide, from the organic acids produced in the first stage (4). The third stage involves methanogenic bacteria which utilize the products of the first two stages, mainly hydrogen, carbon dioxide, and acetate, in the production of methane and carbon dioxide. Hydrogen plays a central role in the degradative pathway as an electron sink for the fermentative reactions carried out by bacteria of the first two stages. Hydrogen acts by controlling the proportions of various products produced by the fermentative bacteria (5,6,18,37,41). The oxidation of reduced

nicotinamide adenine dinucleotide (NADH) with hydrogen production is essential for the degradation of organic matter to proceed. Hydrogen partial pressures are maintained at very low levels by hydrogen-metabolizing methanogenic bacteria (41).

The effects of temperature and retention time on anaerobic fermentation have been studied (7,38). The advantages of thermophilic temperatures (50 to 65°C) include increased reaction rates in relation to the destruction of organic matter and, therefore, reduced capital cost as a result of smaller fermentor size, increased efficiency with respect to the amount of organic matter destroyed, improved solid-liquid separation, and increased destruction of bacterial and viral pathogens.

The addition of oxidants to the anaerobic digestive system may increase the rate and extent with which substrates are degraded by lowering the levels of reduced NAD directly ( $O_2$ ,  $NO_3$ ) or hydrogen partial pressures ( $SO_4$ ).

The inherent problem with the addition of oxygen to the anaerobic digestion system is its toxicity to obligate anaerobes, especially the methanogenic bacteria. Factors, other than super oxide radical and hydrogen peroxide formation, appear to be more important as mechanisms responsible for oxygen toxicity. O'Brien and Morris (33) examined the influence of oxygen on the enzymatic activities of Clostridium acetobutylicum. They found that oxygen dramatically increased the enzymatic activities of NADH oxidase, the enzyme or group of enzymes that converts NADH to NAD by removing the hydrogen of NADH and binding oxygen. Other enzyme activities examined were not affected. They

speculated that NADH oxidase serves as a reductive defense mechanism for the organisms by ridding the environment of limited quantities of oxygen. At high oxygen concentrations, the rate of NADH oxidation exceeds the rate at which it can be supplied by the organisms, and the biosynthetic functions that depend on NADH are interrupted.

Sulfate addition is inhibitory to anaerobic digestion systems. The basis for sulfate inhibition of methanogenesis is unknown. MacGregor and Keeney (29) proposed that the inhibition is due to an increase in oxidation/reduction potential (Eh) of the medium. Capenberg (9) suggested that sulfide produced by sulfate-reducing bacteria inhibits methanogenesis. Martins and Berner (32) have found that methanogenesis in marine sediments does not occur until sulfate is depleted. They speculate that the inhibition of methanogenesis in complex ecosystems in the presence of sulfate is due to a competition for available hydrogen.

Nitrate has also been found to inhibit methanogenesis (2). Studies of the influence of nitrogen oxides on methanogenesis in soils have led to the conclusion that the inhibitory effect observed is due to changes in the oxidation potential and/or substrate competition. Nitrate added to anaerobic ecosystems has been demonstrated to undergo reduction by two pathways (13,22). In denitrification, nitrate is reduced to nitrite and then to nitric oxide, nitrous oxide, and nitrogen gas. In dissimilatory reduction of nitrate to ammonium, nitrate is again first reduced to nitrite before further reduction to ammonium. Dissimilatory nitrate reduction has received little research interest as compared to denitrification (see 25 for most recent review).

Dissimilatory nitrate reduction to ammonium has been described in soils (8,10), freshwater sediments (12), marine sediments (26,36), and the bacteria of several genera (13). In the rumen, dissimilatory reduction of nitrate to ammonium was first studied because of its toxic effects on livestock production. The consumption of feedstocks containing high levels of nitrate may lead to the acute toxicity syndrome known as nitrate poisoning (3). Nitrate is reduced to ammonium in the rumen with nitrite as an intermediate (17,20,27,39). The accumulated nitrite intermediate is absorbed and the syndrome appears when the concentration of blood nitrite is sufficient to cause relatively severe methaemoglobinaemia (39) and circulatory disturbances associated with the vasodilatory effect of the nitrite ion (17). Lewis (28) first showed with washed suspensions of rumen bacteria that 95-100% of the added nitrate was reduced to ammonia. Later, Jones (21) used rumen liquor and showed that, although denitrification occurred, its quantitative significance as a pathway in nitrate reduction in the rumen was small and dissimilatory nitrate reduction of nitrate to ammonium was the major pathway. Dissimilatory reduction of nitrate to ammonium has been reported to account for up to 70% of the nitrate reduced in mesophilic digester sludge (23).

Although the addition of nitrate to anaerobic digestion systems has been reported to inhibit methanogenesis, nitrate addition to the sludge may act to maintain lower levels of NADH. Maintenance of lower levels of NADH could serve to increase the extent of complex organic matter degradation by pulling the fermentative reactions which depend upon electron removal.

This study details the acclimation and establishment of continuous nitrate addition to a thermophilic anaerobic digestion system. The fate of added nitrate is elucidated by analysis of gas composition, ammonium pools in the sludge, and experiments conducted with  $^{15}\text{N}$ -labeled nitrate. The effects of continuous nitrate addition on digester effluent composition and particle size distribution, as well as shifts in microflora, are examined.

The effects of continuous nitrate addition to digester sludge were analyzed as they pertain to single and multi-stage digestion systems.

## MATERIALS AND METHODS

### Digester Construction and Operation

Digesters were constructed from 4 liter aspirator bottles. The aspirator bottles were stoppered with #11.5 butyl rubber stoppers bored for access ports. Digesters were stirred 30 minutes each hour by a bent rod rotated externally with a 100 rpm motor. The wire rod was enclosed in Tygon tubing which was inserted through the stopper and sealed at the bottom. Access ports for feed introduction and gas collection were made of glass tubing attached to amber latex tubing and sealed with pinch clamps. Digesters were placed in 55°C water baths. Effluent was removed through the aspirator spouts, which were attached by rubber tubing to ports inserted through the lower side of the water baths. Sludge volume was maintained at 4 liters. Gas was collected and measured in 10 liter calibrated water displacement vessels above each digester.

Digesters were batch fed daily 16 g of feed in 200 ml of tap water, yielding a hydraulic detention time of 20 days. The feed consisted of 75% bermuda grass and 25% Universal cattle feed (Seminole Brands). Both the bermuda grass and the Universal feed were processed by first reducing particle size in a food processor (Sunbeam), followed by pebble milling (24 hours at 4°C). The processed feed was sifted through an approximately 1.5 mm sieve.

Additions to digesters were made continuously (24 hours/day) with syringe pumps (Harvard Apparatus) with 30 ml plastic syringes fitted

with 1 mm I.D. Tygon tubing connected to 3 inch, 18 ga. needles. Syringes were refilled every 24 hours.

Effluent was removed from the digester by starting the stirring motor 5 minutes prior to sampling to ensure a homogeneous mixture. Approximately 20 ml of effluent were removed and discarded before removing the desired sample. Metabolic activity in the sludge was stopped by the addition of acetonitrile (20% final concentration) or phosphoric acid (2% final concentration).

#### Gas Analysis

Gases were collected by starting the digester stirring motor. A gas collection vessel was constructed by cutting a glass serum tube 5 cm from the bottom and fire polishing the cut. A stopper was inserted into the crimp end and a crimp seal attached. The tube was then filled with saturated sodium chloride, 0.004 M potassium hydrogen phalate, and pH 4.0, and was placed inverted in a filled plastic centrifuge tube (80 ml). The gas exhaust tubing was clamped off and a 20 ga. needle inserted through the digester stopper. The needle was attached with Tygon tubing (1 mm I.D.) to the gas collection vessels. Gas was allowed to flush 5 minutes before collection. Gas samples (200  $\mu$ l) were removed and injected into the gas chromatograph with a 25 ga., 7/8 inch needle attached to a 250  $\mu$ l gas-tight syringe (Hamilton).

Methane, carbon dioxide, hydrogen, and nitrous oxide were determined with a Hewlett Packard Model 5880 gas chromatograph equipped with a thermal conductivity detector and a Porapack Q column (80/100 mesh, 6 ft. x 1/8 in. O.D., Alltech Associates). Detector and oven temperatures were maintained at 145 and 120°C, respectively. Helium

served as carrier. Nitrogen was measured with a Loenco Model 15A gas chromatograph equipped with a thermal conductivity detector and integrator. A Molecular sieve 4A column (80/100 mesh, 6 ft. x 1/8 in. O.D., Alltech Associates) was used with helium as carrier gas. Detector and oven temperatures were maintained at 27°C. Chromatographs were calibrated with high purity gas standards (Matheson).

Gas pressures were determined in stoppered reaction vessels by use of a pressure transducer (Setra Systems) connected to a 20 ga. needle inserted through the stopper. Instantaneous gas production rates were determined using a soap film flowmeter (Hewlett Packard).

Trace oxygen was removed from gas used for flushing sludge head space or medium preparation by passage of the gas over heated (350°C), reduced copper turnings.

#### pH, Volatile Fatty Acid, and Ion Analysis

An Orion Research (Model 701A) Digital Ionalyzer and pencil-type, gel-filled probe (Corning) were used for pH measurements. Before the sludge pH measurements, the probe was incubated in pH 7.0 buffer at 55°C. Sludge was drawn into serum tubes, stoppered, and incubated at 55°C prior to pH measurement. Samples were analyzed within 10 minutes of removal from the digester.

Volatile fatty acids were determined with a Hewlett Packard gas chromatograph (Model 5880) equipped with a flame ionization detector, autosampler, and integrator. Volatile fatty acids were separated using a column (6 ft. x 1/8 in. O.D.) packed with 10% AT-1200 + 1% phosphoric acid on Chromasorb W-AW, 80/100 mesh (Alltech Associates).

Injection port and column temperature were maintained at 145 and 120°C, respectively. Helium served as carrier. Sludge was prepared for analysis by mixing 1:1 with 4% O-phosphoric acid. After thorough mixing, the sample was transferred to 1.5 ml micro-centrifuge tubes (Fisher). The tubes were centrifuged at 12,800 x G at room temperature for 15 minutes with a micro-centrifuge (Eppendorf, Model 5414). The supernatant was transferred to 1 ml auto sampler serum vials (Wheaton), closed with a Teflon-lined crimp cap, and loaded with appropriate standards similarly prepared into the autosampler.

Nitrate and sulfate were determined with a Hewlett Packard liquid chromatograph (Model 1084B) equipped with an autosampler, conductivity detector (Wescan), and integrator. They were separated on an Anion Exclusion Column (Wescan) using 0.004 M potassium hydrogen phalate at pH 3.9 as the eluent at a flow rate of 1.5 ml/minute. Eluent, detector, and oven temperatures were maintained at 45°C. Samples for nitrate and sulfate were prepared by diluting sludge 1:1 with acetonitrile to stop further metabolic activity. After being thoroughly mixed, the samples were further diluted to 1/10 final with distilled water. The diluted sludge was then centrifuged and the clear supernatant transferred to autosampler vials. Nitrite was determined by the colorimetric method as described (14). Sludge supernatant (0.5 ml), prepared with acetonitrile, as previously described, was added to 19 x 150 mm spectrophotometer tubes (Coleman, grade A). To each tube 6.1 ml of distilled water and 0.9 ml of indicator reagent were added. The indicator reagent contained 10% v/v phosphoric acid (85%), 4% w/v sulfanilamide (98.6%, Fisher), and 0.22% w/v N-(1-naphthyl)ethylenediamine dihydrochloride in distilled

water. The reagent was stored in a dark bottle at 4°C. The spectrophotometer tubes were mixed vigorously and allowed to incubate at room temperature for 15 minutes before being read at 540 nm with a Perkin-Elmer (Model 35) spectrophotometer. Nitrite standards were analyzed to construct a standard curve to which experimental nitrite samples were compared.

Ammonium ion plus ammonia was determined with an ammonia electrode (Corning) and an Orion Research Digital Ionalyzer (Model 701A). Sludge samples (2 ml) were pipetted into 250 ml beakers to which 88 ml of distilled water were added. The diluted sludge was stirred on a magnetic mixer. The ammonia electrode was lowered into the solution at an angle to avoid bubbles becoming trapped on the electrode membrane. A solution of 1.0 M NaOH (10 ml) was immediately pipetted into the beaker and the relative millivolt reading recorded after 1.5 minutes. Ammonium ion plus ammonia concentrations were calculated from a standard curve relating concentration to relative millivolts derived from standards prepared from ammonium chloride.

#### Stable Isotope ( $^{15}\text{N}$ -Nitrate) Experiments

For  $^{15}\text{N}$ -label analysis, sludge samples (4 ml) were removed from experimentals and pH was reduced to approximately 4.0 by the addition of 0.3 ml of 10% sulfuric acid, to convert ammonia to stable ammonium ion, in serum tubes. The samples were mixed well and frozen until distillation. Ammonium and/or nitrate (first reduced to ammonium) were distilled as described (24). Sludge ammonium distillates were concentrated to approximately 3 ml by evaporation on a hot plate (Corning)

while flushed with air. Samples were transferred to small vials and analyzed for  $^{15}\text{N}$ -ammonium ratio as described (16). Percent  $^{15}\text{N}$ -label calculations were based on  $^{15}\text{N}$  recovered in ammonium distillation from sludge removed during the experiment versus  $^{15}\text{N}$  recovered from both ammonium plus nitrate distillation at the time of introduction of the label (zero time).

#### Digester Effluent Composition and Particle Size Distribution Analysis

Sludge was analyzed for dry matter and volatile solids. Sludge (200 ml) was removed from each digester into 250 ml flat-bottom boiling flasks. A magnetic stir bar was added and the sludge vigorously mixed on a magnetic mixer. Samples (25 ml) were removed with 25 ml wide-tip pipettes and placed into crucibles (30 ml, Coors) which had been previously heated overnight at 550°C, cooled to room temperature in a desiccator, and weighed (analytical balance, Mettler). The samples were dried overnight at 110°C, cooled to room temperature in a desiccator, and weighed. Samples were then ignited at 550°C for two hours in a muffle furnace. After the samples were cooled to room temperature in a desiccator, they were weighed to constant weight.

Effluent was analyzed for acid detergent fiber (ADF), lignin, and cellulose plus cutin, as described (13).

Effluent particle size distribution was determined by washing 400-1000 ml of effluent through sieves (250, 355, 500, and 710  $\mu\text{m}$  pore size, 8 inch diameter, Fisher) and rinsing the trapped material into tared aluminum weigh boats. The samples were dried overnight at 110°C, cooled to room temperature in a desiccator, and weighed to constant weight on an analytical balance.

Experiments conducted with sludge external to the digesters were made in 150 ml serum bottles (Wheaton). Sludge was removed from the digesters 2 hours after feeding and vigorously mixed in flat-bottom boiling flasks on a magnetic stirrer while flushing with oxygen-free  $N_2/CO_2$  (80/20% mixture, Matheson). Either 25 or 100 ml aliquots were pipetted into 150 ml serum bottles while the bottles were flushed with the same oxygen-free gas. Serum bottles were closed with butyl rubber stoppers and aluminum crimp caps attached. Additions to the sludge were made through the stopper with 20 ga. needles attached to 1 or 5 ml syringes. The samples were incubated at 55°C with shaking, with either a shaking water bath (New Brunswick, Model G-76) or in an incubator on an orbital shaker (Junior Orbit, Lab Line). Gas samples were removed with 25 ga. needles attached to 250  $\mu$ l gas-tight syringes and analyzed as previously described. Serum bottle gas pressure was determined as previously described. Sludge was removed with 20 ga. needles attached to 1, 5, or 10 ml syringes.

#### Enumerations of Digester Microflora

Digester sludge was analyzed for viable cell numbers of several groups of microorganisms. Digester effluent (100 ml) was removed 24 hours after feeding and blended in a semi-micro blender (Eberbach, Waring) for 30 seconds to break up microbial masses. The sludge was flushed with oxygen-free  $N_2/CO_2$  (80/20%) gas during blending. The blended sludge was subjected to ten 10-fold dilutions in 9 ml anaerobic dilution blanks. Anaerobic dilution blanks consisted of basal medium under an oxygen-free  $N_2/CO_2$  gas phase. Anaerobic basal medium was

prepared in serum tubes by the methods of Hungate (19), as described by Balch et al. (1). Basal medium composition was as follows: trace vitamin solution (40) 10 ml; trace mineral solution (40) 10 ml;  $\text{NH}_4\text{Cl}$  1.0 g;  $\text{KH}_2\text{PO}_4$  2.0 g;  $\text{NaHCO}_3$  5.0 g; resazurin 0.001 g; cysteine HCl 0.5 g; clarified digester sludge 10 ml; distilled water to 1000 ml. One milliliter of each dilution was injected into 25 ml of tempered agar medium—basal medium, additions, and 2% noble agar (Difco)—contained in 150 ml serum bottles. Bottles were rolled in a level pan containing cool water until the agar solidified. The roll bottles were incubated at 55°C, inverted with either a  $\text{N}_2/\text{CO}_2$  or  $\text{H}_2/\text{CO}_2$  (both 80/20%) gas phase. Colonies were examined and counted with an Olympus stereo microscope after 1 and 2 weeks of incubation.

Cellulose degraders were enumerated in basal medium plus cellulose (1% final concentration). Cellulose fibers used in the medium preparation were Whatman #1 filter paper blended in a standard blender (Waring) with distilled water. The cellulose slurry was centrifuged in 250 ml plastic screw-capped tubes at 7000 rpm, 4°C, for 30 minutes (Sorvall RC-2 centrifuge, GSA rotor). The supernate was discarded and the cellulose pellet was washed twice more in this manner after it was resuspended with 200 ml of distilled water. The final cellulose pellet was resuspended in 100 ml of distilled water and frozen in a 1 liter vacuum flask in an acetone/dry-ice bath. The cellulose was lyophilized (Virtus lypholyzer) to dryness. Colonies which degraded the opaque cellulose fibers, producing clear zones, were counted.

Hydrogen-metabolizing methanogenic bacteria were cultivated in basal medium under a  $\text{H}_2/\text{CO}_2$  gas phase. Hydrogen-oxidizing, carbon

dioxide-reducing methanogens were enumerated by counting colonies which autofluoresced when illuminated with a 420 nm light (11).

Acetate-metabolizing methanogenic bacteria were enumerated by counting colonies which autofluoresced when illuminated with a 420 nm light in basal medium containing 4.0 g/l sodium acetate under a  $N_2/CO_2$  gas phase.

Sulfate-reducing bacteria oxidizing hydrogen were enumerated. The basal medium was supplemented with sodium sulfate (1 g/l), and prior to inoculation 0.25 ml of a sterile  $FeSO_4 \cdot 7H_2O$  solution (0.5 g/l final concentration) was added. Colonies were counted which produced a black precipitate resulting from  $FeS$  formation from the sulfide produced.

Nitrate reducers were enumerated by the incorporation of 100  $\mu$ moles of  $NaNO_3$ /ml medium, glucose (3.0 g/l final), and yeast extract (3.0 g/l final) to the basal medium. The medium surrounding colonies was tested for the presence of nitrate by the addition of a few drops of a mixture of 1:2 diphenylamine : concentrated sulfuric acid (35). The production of a dark blue-black color indicates nitrate presence. Colonies were found to be exclusively nitrate-reducing microorganisms.

## RESULTS

### Nitrate Introduction to the Thermophilic Anaerobic Digestion System

Thermophilic anaerobic digesters were constructed and maintained. Initially, all digesters were within 5% of each other in the parameters measured. The addition of nitrate to one digester was begun slowly by pumping nitrate solutions over various time intervals (Figure 1). The effects of the pumped nitrate on digester parameters were monitored. Total gas production was the major parameter used in assessing digester condition.

Initially, when small total amounts of nitrate were pumped into the digester, gas production fell sharply. Recovery was slow. Upon subsequent additions, gas production recovered faster and greater total amounts of nitrate were required to effect the same inhibition observed earlier. A lower concentration of nitrate was then pumped over a longer period of time beginning at day 30. It was determined that a pump rate of 0.4  $\mu$ moles nitrate/ml sludge/hour could be maintained with a resulting 20-30% inhibition of gas production. When an attempt was made to increase the pump rate to 0.5, the gas production dropped to 50-60% of that of the control. When the pump was then shut off for over 15 days, gas production rate recovered to essentially 100% of that of the control. The pump rate of 0.4  $\mu$ moles  $\text{NO}_3$ /ml sludge/hour was then again established. During this time of digester acclimation, volatile fatty acids and ammonia pools were also monitored (Figure 2). Acetate accumulation

showed the same acclimation to increases in nitrate addition as did the gas production, and after day 30 did not appear to be affected by nitrate addition until a pump rate of 0.5 was used. Ammonia pools increased proportionally with the total nitrate pumped.

The digester was maintained at a pump rate of  $0.4 \mu\text{moles NO}_3/\text{ml}$  sludge/hour, and parameters were monitored to determine whether a stable constant inhibition could be obtained (Figure 3). Over a 320 day period the total gas production was maintained at 70% of the control's. The acetate pool increased and leveled off at  $40 \mu\text{moles/ml}$  sludge (Figure 4). The ammonia pools were proportional to the nitrate added and leveled off at  $195 \mu\text{moles/ml}$  sludge.

The measured ammonia pools corresponded to pools predicted from 100% conversion of nitrate to ammonium (Figure 5). Two more digesters were initiated, one with sodium nitrate added and one with ammonium chloride added. Table 1 lists the parameters measured after all digesters had stabilized. The digester pumped ammonium chloride most resembled the control digester in all parameters. Gas production was only slightly inhibited and pH was slightly lower. The calcium nitrate digester was less inhibited than the sodium nitrate-pumped digester as shown by higher gas production and lower volatile fatty acid pools.

Analysis of Digester Gas Production and Composition,  
Volatile Fatty Acids, Ammonium,  
and pH After Feeding

Gas Production

Parameters measured to determine digester performance were analyzed over a 24 hour period following feeding. Cumulative gas production is shown in Figure 6. After 24 hours the nitrate-pumped

Table 1. Effects on digester parameters measured of continuous addition of various solutions to thermophilic anaerobic digesters<sup>a</sup>

| Digester designation                                            | E-1        | D-1                               | D-2               | F-2                |
|-----------------------------------------------------------------|------------|-----------------------------------|-------------------|--------------------|
| Solution pumped <sup>b</sup>                                    | None       | Ca(NO <sub>3</sub> ) <sub>2</sub> | NaNO <sub>3</sub> | NH <sub>4</sub> Cl |
| % of control gas production                                     | 100        | 71.8 ± 1.6                        | 51.3 ± 2.0        | 92.7 ± 6.2         |
| pH                                                              | 7.2 ± 0.05 | 7.6 ± 0.08                        | 7.8 ± 0.05        | 7.0 ± 0.15         |
| Volatile fatty acid pools                                       |            |                                   |                   |                    |
| Acetate <sup>c</sup>                                            | 1.6 ± 0.8  | 58.1 ± 16.1                       | 107.8 ± 35.0      | 1.2 ± 0.7          |
| Propionate <sup>c</sup>                                         | 1.1 ± 2.4  | 10.2 ± 1.2                        | 12.5 ± 1.5        | 1.3 ± 1.4          |
| Ammonium (NH <sub>3</sub> +NH <sub>4</sub> ) pools <sup>c</sup> | 31.3 ± 2.3 | 194.0 ± 4.2                       | 192.2 ± 3.8       | 195.0 ± 4.8        |

<sup>a</sup>Data represent measurements recorded over a 30 day period in which all digesters remained stable.

<sup>b</sup>Pump rates were maintained at 0.4 µmoles NO<sub>3</sub> or NH<sub>4</sub>/ml sludge/hour.

<sup>c</sup>Pools reported in micromoles/ml sludge.

digester produced 70% of the control digester gas. Rates of gas production for control and nitrate-pumped digesters are shown in Figure 7. The inhibition of the rate of gas production varies over the 24 hour period following feeding. At 4, 8, 12, 16, 20, and 24 hours, the nitrate-pumped digester is 63, 62, 60, 70, 80, and 81%, respectively, of the control digester gas production rate.

#### Gas Composition

Figure 8 depicts the changes in composition of gas produced after feeding. After feeding (zero time) the percentage of methane drops and is replaced by increased carbon dioxide levels. At 2 hours the methane and carbon dioxide percentages are equivalent. After 2 hours the percentage of methane in the gas increases with a subsequent decrease in the percentage of carbon dioxide. At 22 hours the percentage of methane in the control digester is 59 compared to 54 for the nitrate-pumped digester.

#### Volatile Fatty Acids in the Sludge

Volatile fatty acid pools monitored after feeding are shown in Figure 9. Initial acetate pools are higher in the nitrate-pumped digester (18  $\mu$ moles/ml sludge) than in the control digester (3  $\mu$ moles/ml sludge). In both digesters acetate levels increase after feeding to a maximum level after 8 hours. The subsequent degradation of acetate is slower in the nitrate-pumped digester than in the control. Propionate levels are also initially higher in the nitrate-pumped digester (10 versus 3.5), but levels appear to remain relatively constant after feeding in both digesters.

### Ammonium Ion Pools in the Sludge

Ammonium pools ( $\text{NH}_3 + \text{NH}_4$ ) were monitored after feeding (Figure 10). Ammonium pools remained constant and at the same levels in the ammonium chloride-pumped digester as in the two nitrate-pumped digesters ( $\text{Ca}(\text{NO}_3)_2$ ,  $\text{NaNO}_3$ ) over the 24 hours after feeding. Ammonium pools were lower in the control digester but they also remained constant after feeding.

### Sludge pH Changes

The pH of the sludge was determined after feeding (Figure 11). The initial pH units of the control, calcium nitrate, and sodium nitrate digesters were 7.1, 7.4, and 7.5, respectively. After feeding, the pH dropped in all digesters 0.1-0.2 pH units but recovered to initial pH readings after 6-8 hours. Digester pH remained relatively constant throughout the remaining 24 hours.

### Inhibition Resulting from Nitrate, Nitrite, and Ammonium Addition

The inhibition resulting from nitrate, nitrite, and ammonium addition to sludge was investigated (Figures 12-17). Figure 12 shows the inhibition of control sludge upon the addition of various concentrations of sodium nitrate. The addition of 1.0  $\mu\text{moles NO}_3/\text{ml}$  sludge has no effect, whereas increases of from 2 to 10  $\mu\text{moles NO}_3/\text{ml}$  sludge increase the inhibition to 60% of that of the control after 48 hours. Increases in nitrate addition above 10  $\mu\text{moles NO}_3/\text{ml}$  sludge do not appear to increase inhibition but do delay the recovery of gas production in the sludge. Nitrite is a greater inhibitor of control sludge gas production (Figure 13). Inhibition caused by the addition of 1 and

2  $\mu\text{moles NO}_2/\text{ml}$  sludge is short-lived and recovery begins after 24 hours. Upon the addition of 5, 10, and 20  $\mu\text{moles NO}_2/\text{ml}$  sludge, the inhibition increases to a maximum of 45% of the control gas production with 20  $\mu\text{moles NO}_2/\text{ml}$  sludge. Figure 14 shows the effects of ammonium addition to control digester sludge. Throughout the range of 1 to 20  $\mu\text{moles NH}_4/\text{ml}$  sludge, inhibition of gas production is not evident, within the precision of the measurements.

In inhibition studies conducted with nitrate-pumped digester sludge (Figure 15), nitrate additions of 1-5  $\mu\text{moles/ml}$  sludge were not inhibitory. The inhibition resulting from the addition of 10 and 20  $\mu\text{moles NO}_3/\text{ml}$  sludge appeared to occur in two steps. Initial sludge gas production was 85 and 75% of control gas production compared to a second inhibition of 74 and 40%, respectively. The higher the amount of added nitrate, the greater the secondary inhibition and lag before recovery of gas production by the sludge. The inhibition of nitrate-pumped digester sludge by nitrite is shown in Figure 16. The addition of 2-20  $\mu\text{moles NO}_2/\text{ml}$  sludge resulted in a comparable inhibition at 196 hours (38% of the control gas production). Sludge recovery was faster in most cases with a lower initial nitrite addition. The effects of added ammonium to nitrate-pumped digester sludge are shown in Figure 17. Essentially, no inhibition was observed upon addition of 1-20  $\mu\text{moles NH}_4/\text{ml}$  sludge.

#### Nitrate Reduction Kinetics

Figure 18 shows the reduction of added nitrate and its effect on gas production in control digester sludge. Nitrate is reduced after a short lag (2 hours), and reduction is complete after 9.5 hours.

Nitrite is an intermediate in the reduction of nitrate to ammonium, and at least 33% of the nitrate passes through nitrite. The reduction of nitrite is complete after 18.5 hours. Gas production is maximally inhibited at 3.5 hours when the majority of the 20  $\mu\text{moles NO}_3/\text{ml}$  sludge is still present and before nitrite accumulates. Gas production in the control sludge began recovery after 3.5 hours, remained low from 7 through 18 hours (during  $\text{NO}_2$  appearance and subsequent reduction), and began recovery again after 18 hours. Nitrate-pumped digester sludge was also assayed for reduction of a 20  $\mu\text{moles NO}_3/\text{ml}$  sludge addition (Figure 19). Nitrate reduction occurs without a lag and is complete after 7.5 hours. Nitrite accumulates faster and constitutes at least 66% of the nitrate reduced to ammonium. Subsequent nitrite reduction was not complete after 26 hours. Gas production was maximally inhibited when high concentrations of nitrate (18  $\mu\text{moles/ml}$  sludge) were still present and when low nitrite accumulation ( $<2\mu\text{moles/ml}$  sludge) was observed. Gas production began recovery after 3.5 hours, but after 7.5 hours (at the point of maximum  $\text{NO}_2$  accumulation) inhibition was again observed.

#### Nitrite Reduction Kinetics

The rate of nitrite reduction was determined for control, nitrate-pumped, and ammonium-pumped digester sludge (Figure 20). Sludge from all three digesters reduced nitrite without a lag. Control sludge reduced the added nitrite within 14 hours of addition. Ammonium-pumped sludge reduced nitrite at rates identical to those of control sludge initially, but reduction was slower after 4 hours. The nitrate-pumped digester sludge reduced nitrite at a faster rate than that of either

the control or the ammonium-pumped digester sludge, initially. Similar to the ammonium-pumped digester sludge, it appeared to experience a saturation point (at 5.5 hours) at which time further reduction of nitrite was slower. Figure 21 shows gas production in the nitrite reduction experiment expressed as percentages of untreated control sludge gas production. Control sludge was inhibited to a maximum of 23% of the untreated control, but recovery began after 10 hours. The ammonium-pumped digester sludge was inhibited the greatest, with a maximum inhibition of 14% of the control's. Recovery of gas production was not evident after 22 hours of incubation. Sludge from a nitrate-pumped digester was the least inhibited (34% maximum), with recovery of gas production beginning after 4 hours.

#### Nitrous Oxide Reduction

The ability of either control or nitrate-pumped digester sludge to carry out denitrification was examined. Figure 22 shows the depletion of added nitrous oxide, an intermediate in denitrification of nitrate, by control and nitrate-pumped digester sludge. Added nitrous oxide was not metabolized by control digester sludge over a 40 day period. Nitrate-pumped digester sludge began metabolizing added nitrous oxide after 4 days, and depletion was complete after 9 days. Although the nitrogen content of the gas phase was not quantitated, increases in the  $N_2$  percentage of the gas phase were observed. This is consistent with the accepted pathway in denitrification of reduction of nitrous oxide to nitrogen gas under reduced conditions (25).

### Stable Isotope Experiments

The conversion of nitrate to ammonium was also established by the addition of  $^{15}\text{N}$ -labeled nitrate to sludge from the nitrate acclimated digester (Figure 23). Recovery of the  $^{15}\text{N}$  label was calculated as the percentage of the amount added. The  $^{15}\text{N}$  label recovered was proportional to the ammonium pool increases over the control ammonium pool. Maximum recovery of 97.8% of the added  $^{15}\text{N}$  label added as nitrate was detected in the ammonium pools.

### Effluent Analysis and Particle Size Distribution

The effluents from control and nitrate-pumped digesters were compared with respect to percentages of dry matter, volatile solids, acid detergent fiber (% ADF), lignin, and cellulose + cutin. The results, shown in Table 2, indicate higher percentages of dry matter and volatile solids and lower percentages of ADF, Lignin, and cellulose + cutin in nitrate-pumped digester sludge as compared to control sludge. The effluent sludge was also analyzed for particle size distribution (Figure 24). Nitrate-pumped digester sludge contained 85% of the control weight in large 710 micrometer particles. Nitrate-pumped sludge contained 111, 109, and 106% of the control sludge weight in 250, 355, and 500 micrometer particles, respectively.

### Enumerations of Microflora in Digester Sludge

Control and nitrate-pumped digesters were compared with respect to microflora by the enumeration of characteristic groups of microorganisms. Table 3 lists the relative numbers of various groups of microorganisms in control and nitrate-pumped digester sludge.

Table 2. Analysis of composition of effluent from control and nitrate-pumped digesters<sup>a</sup>

| Digester designation           | E-1              | D-1                                            |
|--------------------------------|------------------|------------------------------------------------|
| Solution pumped                | None             | $\text{Ca}(\text{NO}_3)_2$                     |
| Pump rate                      |                  | 0.4 $\mu\text{moles NO}_3/\text{ml sludge/hr}$ |
| % dry matter <sup>b</sup>      | $3.26 \pm 0.15$  | $3.35 \pm 0.11$                                |
| % volatile solids <sup>b</sup> | $81.64 \pm 0.39$ | $88.13 \pm 0.59$                               |
| % acid detergent fiber         | $32.21 \pm 2.03$ | $29.35 \pm 1.05$                               |
| % lignin                       | $8.86 \pm 0.54$  | $8.13 \pm 0.42$                                |
| % cellulose + cutin            | $19.84 \pm 1.09$ | $17.26 \pm 0.99$                               |

<sup>a</sup>Values represent data from two analyses conducted on sludge collected approximately 3 weeks apart.

<sup>b</sup>Calculations were based on weights corrected for calcium input.

Table 3. Enumeration of various groups of microorganisms in control and nitrate-pumped digester sludge<sup>a</sup>

| Digester designation | E-1               | D-1                                           |
|----------------------|-------------------|-----------------------------------------------|
| Solution pumped      | None              | Ca(NO <sub>3</sub> ) <sub>2</sub>             |
| Pump rate            |                   | 0.4 $\mu$ moles NO <sub>3</sub> /ml sludge/hr |
| Cellulose degraders  | $5.6 \times 10^6$ | $6.5 \times 10^6$                             |
| Sulfate reducers     | $2.1 \times 10^7$ | $7.4 \times 10^6$                             |
| Methanogens          |                   |                                               |
| Hydrogen             | $1.1 \times 10^9$ | $1.4 \times 10^7$                             |
| Acetate              | $1.7 \times 10^5$ | $6.4 \times 10^3$                             |
| Nitrate reducers     | $3.9 \times 10^8$ | $6.9 \times 10^8$                             |

<sup>a</sup>All enumerations represent the average of duplicate roll tubes. Enumerations were conducted on three occasions approximately 3 weeks apart with data comparison within 10%.

Nitrate-pumped digester sludge contains higher numbers of cellulose-degrading and nitrate-reducing bacteria, with decreased numbers of hydrogen- and acetate-utilizing methanogens and hydrogen-oxidizing, sulfate-reducing bacteria.

Figure 1. Percent of control gas production during acclimation of a digester to nitrate addition.

The 24 hour gas production was measured from calibrated water displacement reservoirs and plotted as a percent of the control (untreated) digester gas production. Gas production is indicated by solid lines and open circles. Nitrate addition rate and duration of addition are indicated by bars.

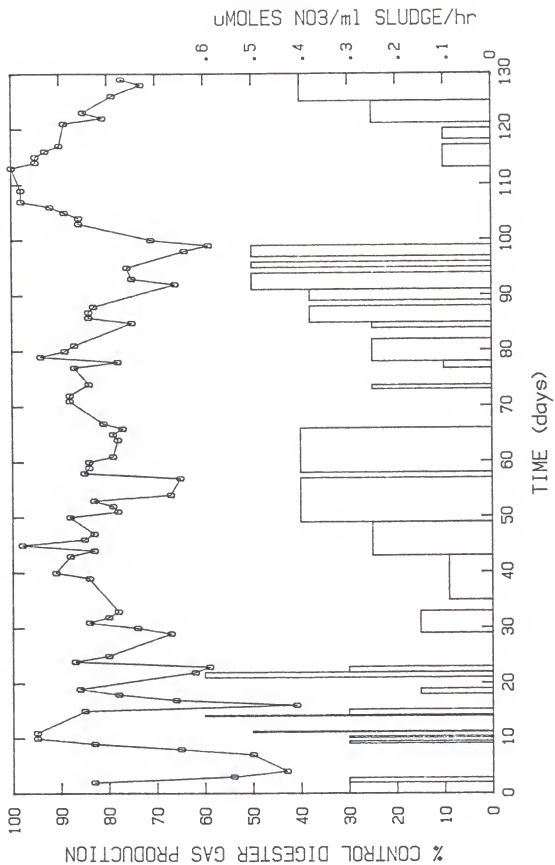


Figure 2. Acetate and ammonium pools in the sludge during acclimation of a digester to nitrate addition.

Micromoles acetate or ammonium ( $\text{NH}_3+\text{NH}_4$ ) per milliliter sludge were determined 24 hours after feeding as described in Materials and Methods. Acetate pools are indicated by open circles and dashed lines. Ammonium pools are indicated by open circles and solid lines. Nitrate addition rate and duration of addition are indicated by bars.

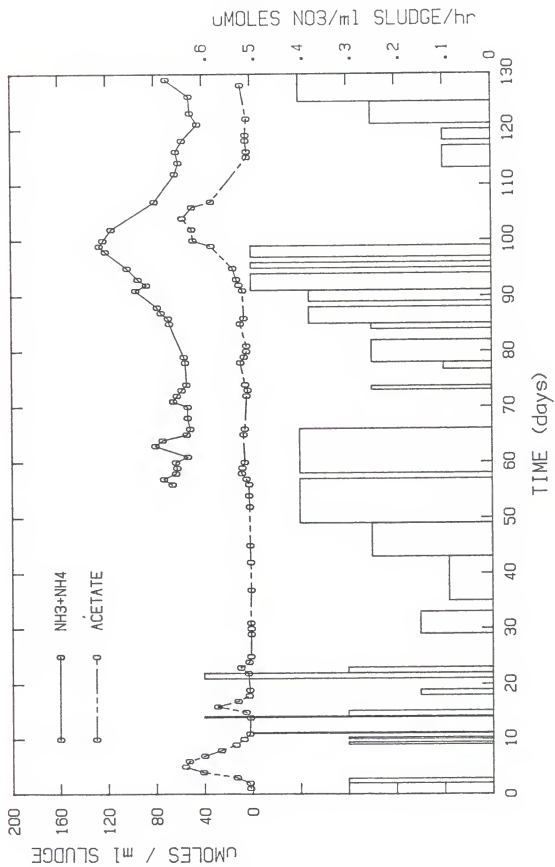


Figure 3. Percent of control gas production during establishment of a continuous nitrate-pumped digester.

The 24 hour gas production was measured from calibrated water displacement reservoirs and plotted as a percent of the control (untreated) digester gas production. Gas production is indicated by solid lines and open circles. Nitrate addition rate and duration of addition are indicated by bars.

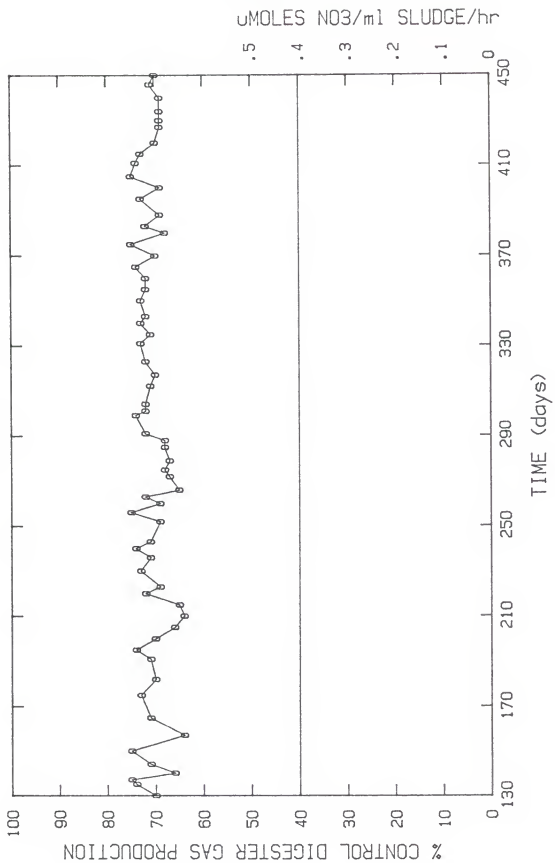


Figure 4. Acetate and ammonium pools in the sludge during establishment of a continuous nitrate-pumped digester.

Micromoles acetate or ammonium ( $\text{NH}_3+\text{NH}_4$ ) per milliliter sludge were determined 24 hours after feeding as described in Materials and Methods. Acetate pools are indicated by open circles and dashed lines. Nitrate addition rate and duration of addition are indicated by bars.

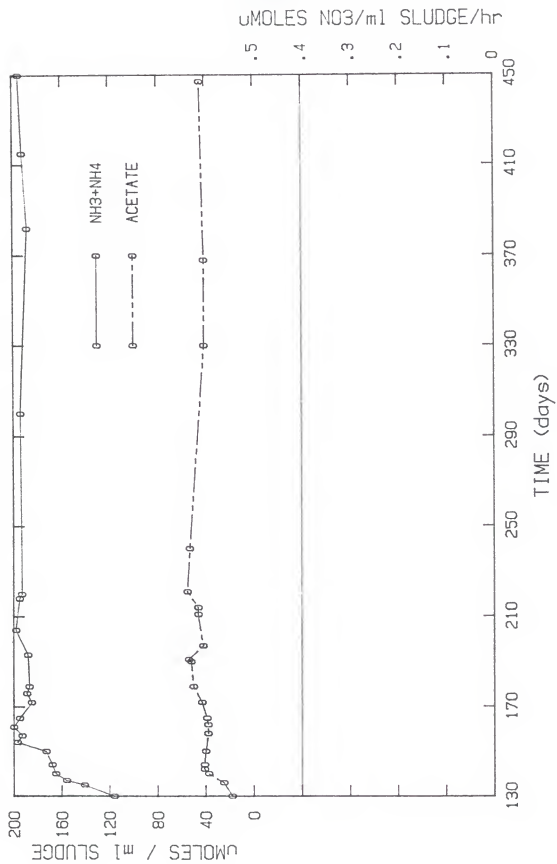


Figure 5. Measured and predicted ammonium pools in the digester-pumped 0.4  $\mu\text{moles NO}_3/\text{ml}$  sludge/hour.

Ammonium pools were measured daily (every 24 hours) immediately prior to feeding as described in Materials and Methods.

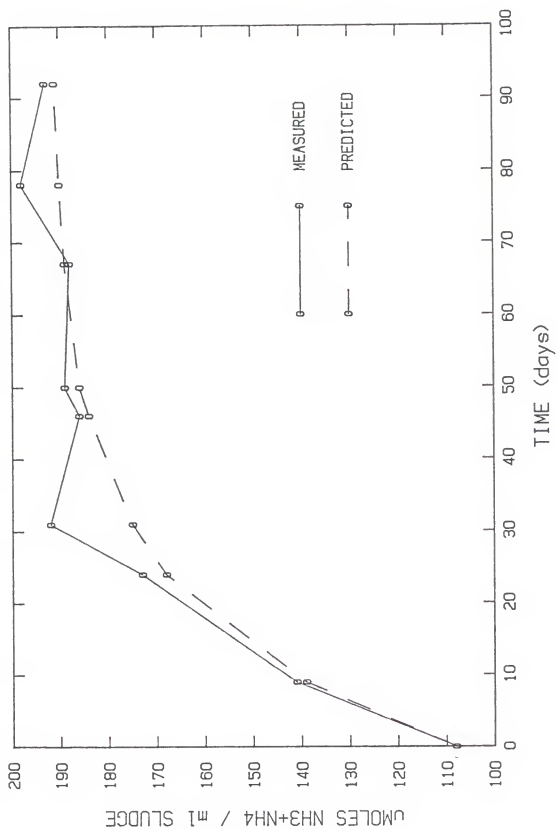


Figure 6. Cumulative gas production in control and nitrate-pumped digesters after feeding. Gas production was measured from calibrated water displacement reservoirs.

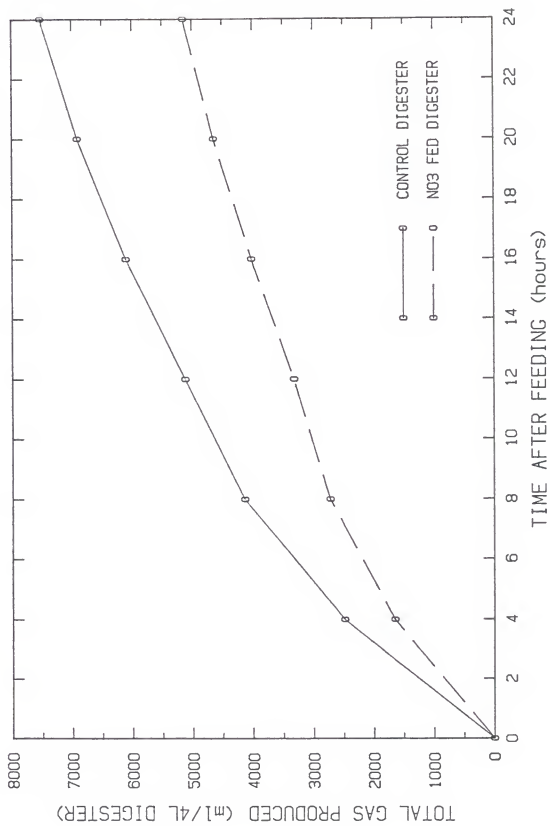


Figure 7. Rate of gas production in control and nitrate-pumped digesters after feeding.

Gas production rates were calculated from total gas production measurements from Figure 6.

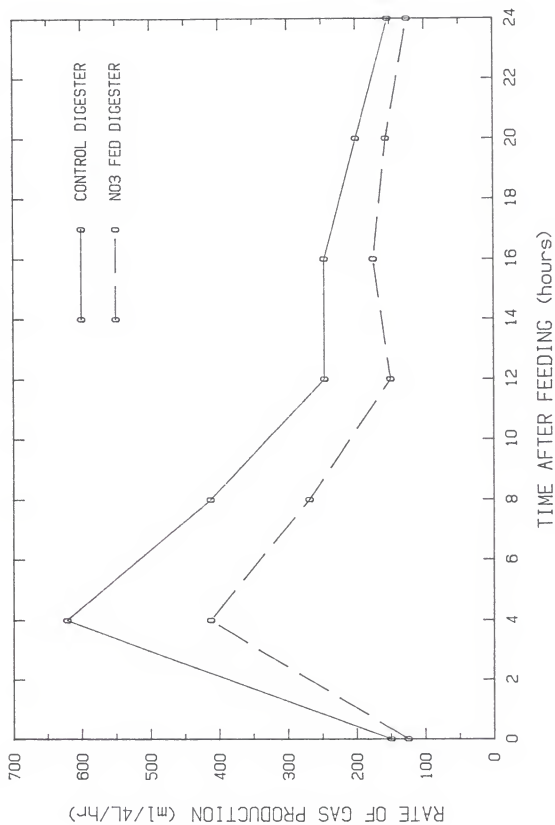


Figure 8. Composition of gas produced from control and nitrate-pumped digesters after feeding. Gas was removed and collected, and percentages methane and carbon dioxide were determined as described in Materials and Methods.

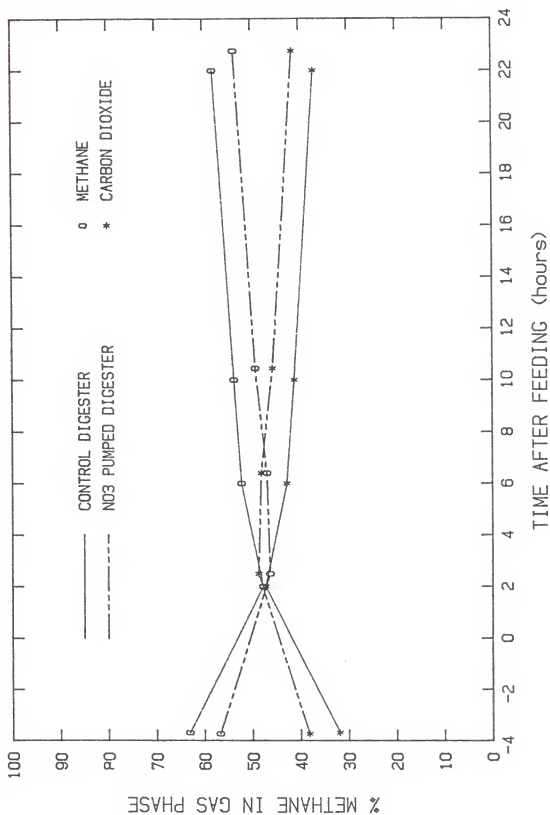


Figure 9. Volatile fatty acid pools, acetate, and propionate in sludge from control and nitrate-pumped digesters after feeding.

Sludge was removed and volatile fatty acids were measured as described in Materials and Methods.

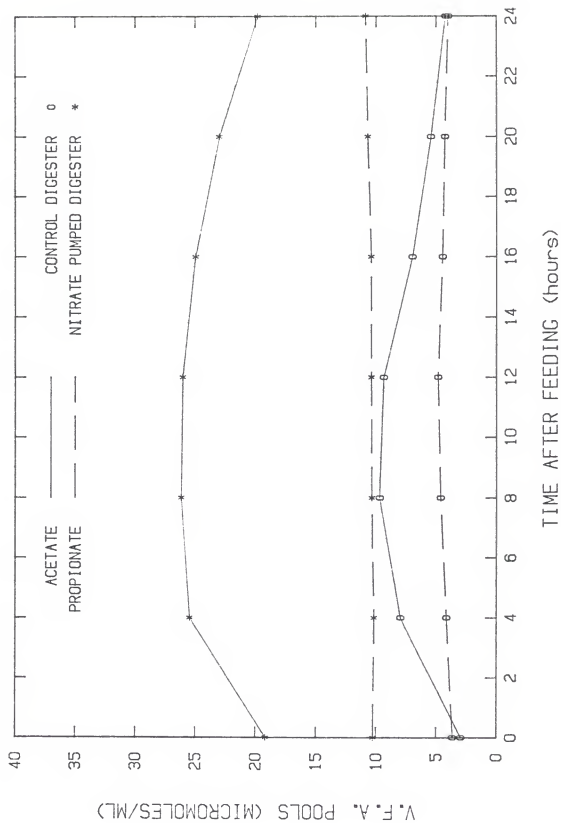


Figure 10. Ammonium ( $\text{NH}_3 + \text{NH}_4$ ) pools measured in digester sludge after feeding.

Sludge was collected and ammonium measured as described in Materials and Methods.

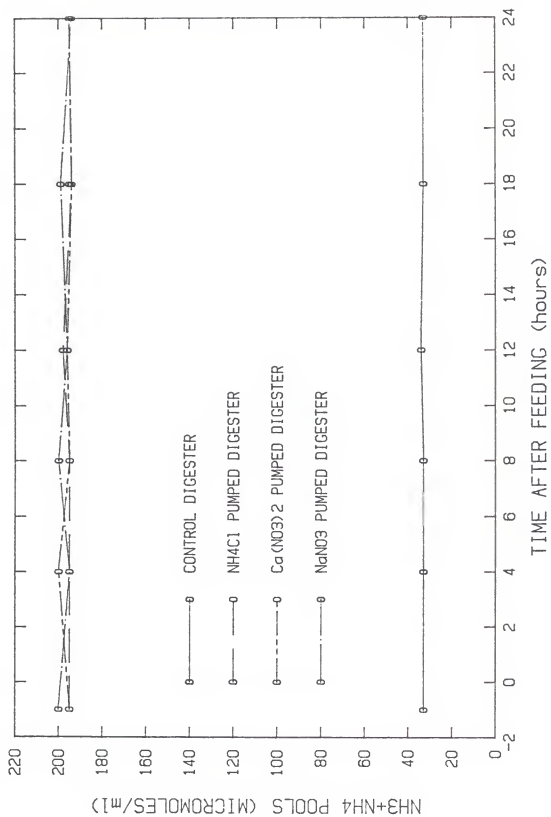


Figure 11. pH measurements in sludge from control and nitrate-pumped digesters after feeding.  
pH measurements were conducted as described in Materials and Methods with special care concerning equilibration of the pH probe to 55°C.

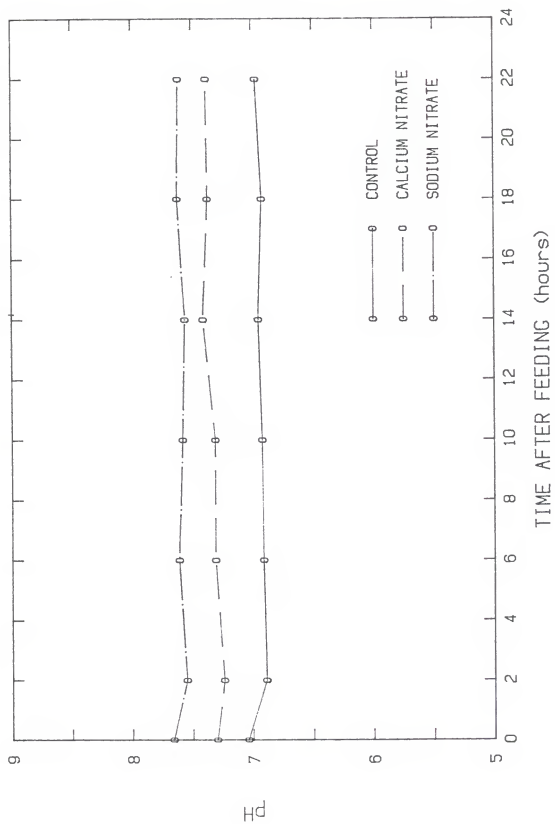


Figure 12. Inhibitory effects of various concentrations of added nitrate on gas production in the control digester sludge.

One milliliter additions of various concentrations of sodium nitrate were added to 25 ml control digester sludge in 150 ml serum bottles. The control upon which experimental results were based received 1 ml distilled water. Experimental bottles were incubated with shaking in a 55°C incubator. Gas production was measured as described in Materials and Methods. Accumulated gas was released after pressure measurements. Points represent the average of duplicate experimental determinations.

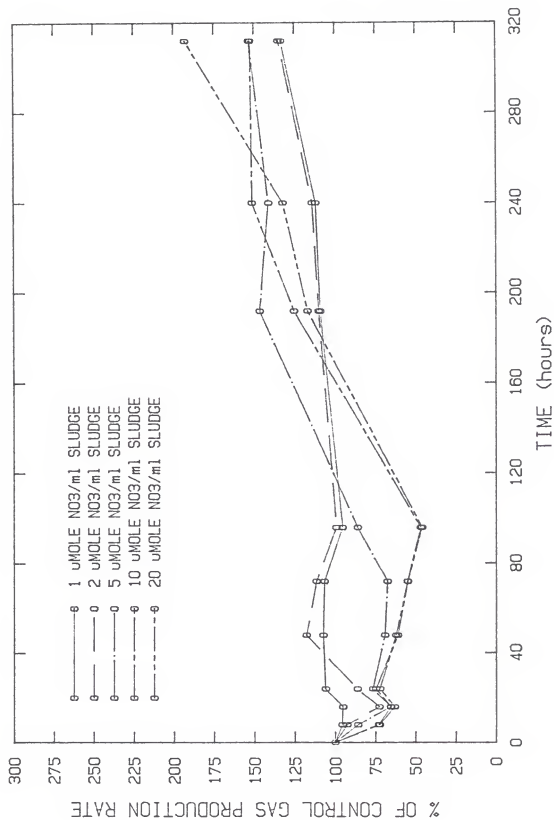


Figure 13. Inhibitory effects of various concentrations of added nitrite on the gas production in control digester sludge.

Procedures were as described in Figure 12.

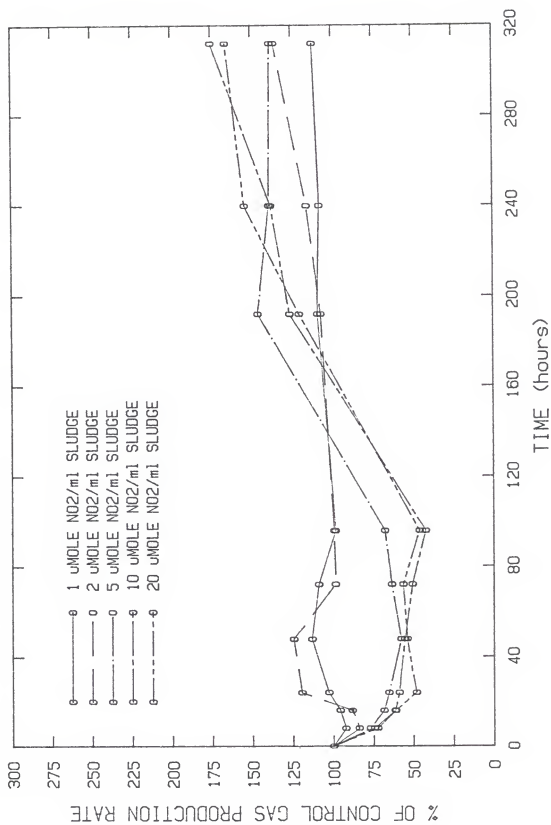


Figure 14. Effects of various concentrations of added ammonium on gas production in control digester sludge.

Procedures were as described in Figure 12.

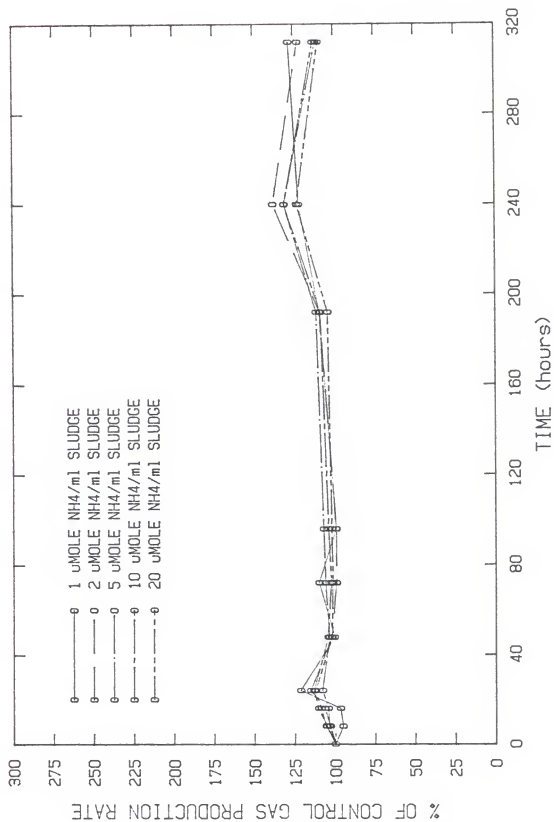


Figure 15. Inhibitory effects of various concentrations of added nitrate on gas production in nitrate-pumped digester sludge.

Procedures were as described in Figure 12.

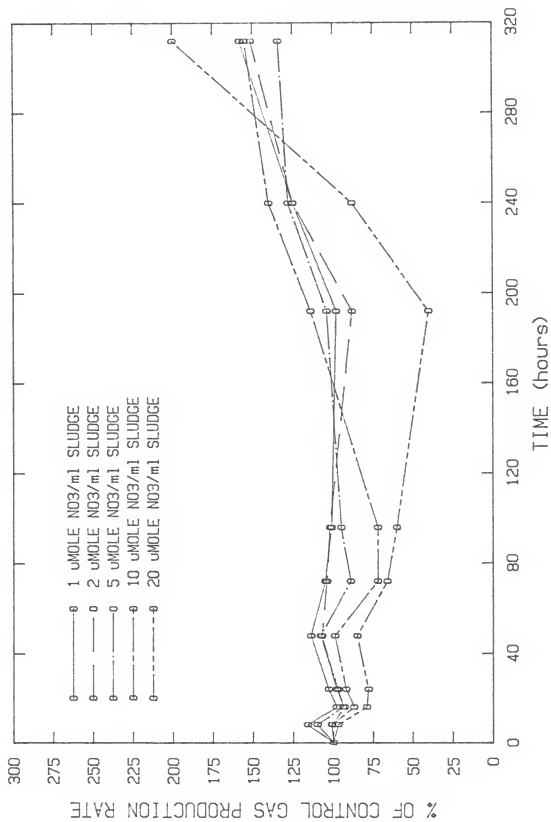


Figure 16. Inhibitory effects of various concentrations of added nitrite on the gas production in nitrate-pumped digester sludge.

Procedures were as described in Figure 12.

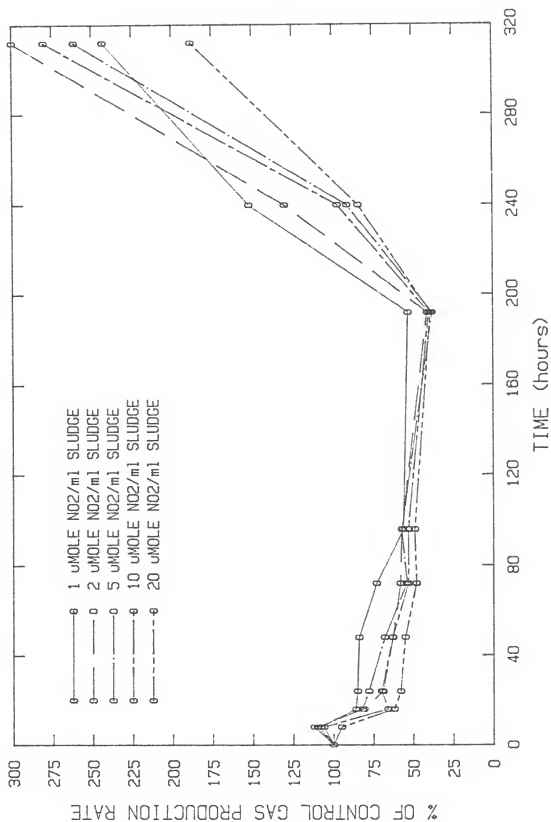


Figure 17. Effects of various concentrations of added ammonium on the gas production in nitrate-pumped digester sludge.

Procedures were as described in Figure 12.

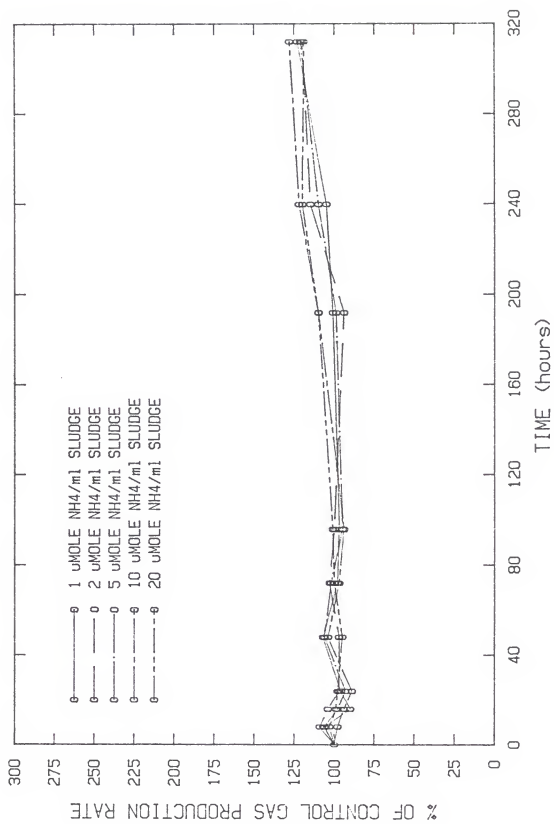


Figure 18. Effects on control digester sludge of a nitrate addition.

A 1 ml addition of sodium nitrate equal to 20  $\mu$ moles  $\text{NO}_3/\text{ml}$  sludge was added to 100 ml of control digester sludge in 150 ml serum bottles. Serum bottles were incubated with shaking in a shaking water bath at 55°C. Gas production in the sludge was measured as described in Materials and Methods with accumulated gas released after each measurement. Sludge was removed and nitrate and nitrite determined as described in Materials and Methods. Points represent the average of duplicate determinations.

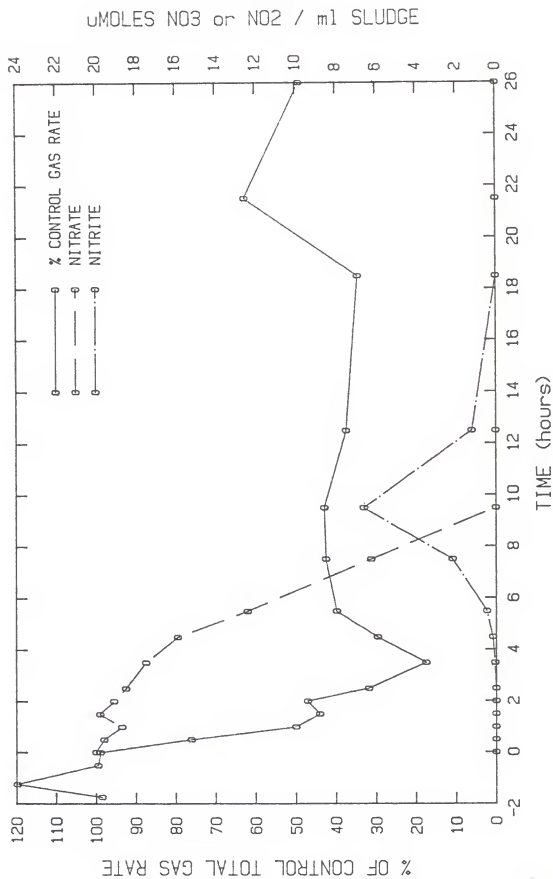


Figure 19. Effects on nitrate-pumped digester sludge of a nitrate addition.  
Procedures were as described in Figure 18.

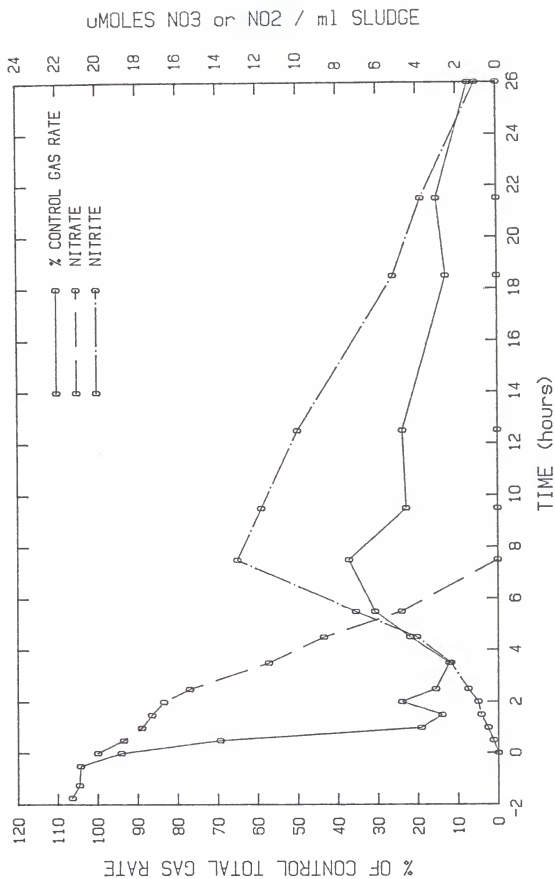


Figure 20. Reduction of added nitrite by digester sludge.

Procedures were essentially as described in Figure 18 except that sodium nitrite was added to the sludge.

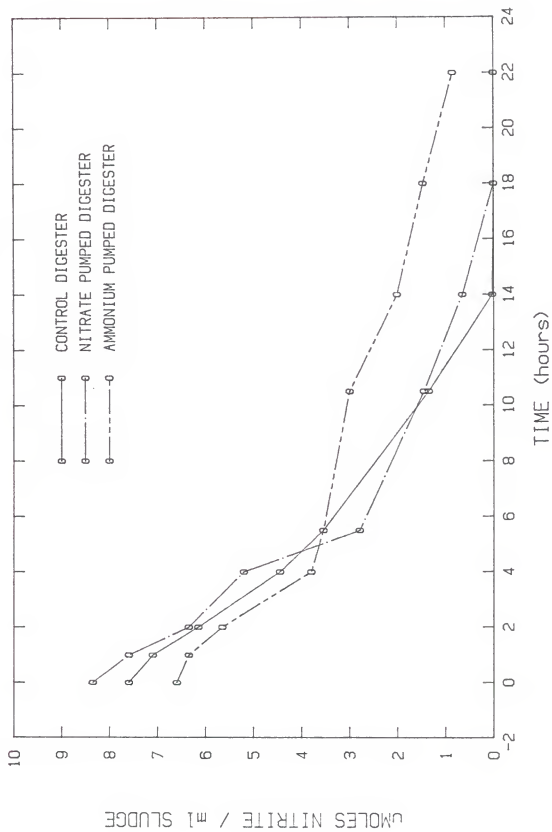


Figure 21. Effects of added nitrite on digester sludge gas production.

Procedures were essentially as described in Figure 18 except that sodium nitrite was added to the sludge.

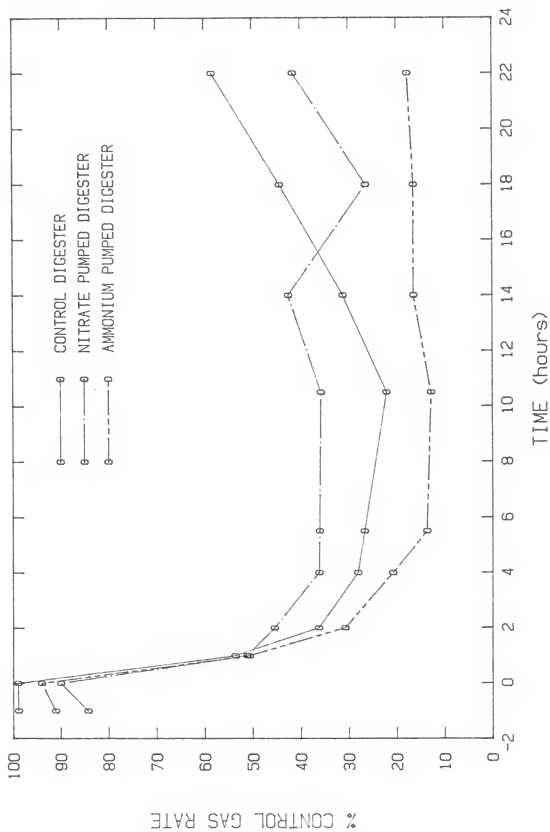


Figure 22. Reduction of added nitrous oxide by control and nitrate-pumped digester sludge.

Twenty-four milliliters sludge aliquots were added to 150 ml serum bottles. Nitrous oxide totaling approximately 10% of the gas phase was added. The experimental bottles were incubated in a 55°C incubator with shaking. Gas production and nitrous oxide composition in the gas phase were measured as described in Materials and Methods. Control bottles consisted of killed sludge (addition of 20% acetonitrile final concentration). Points represent the average of triplicate determinations.

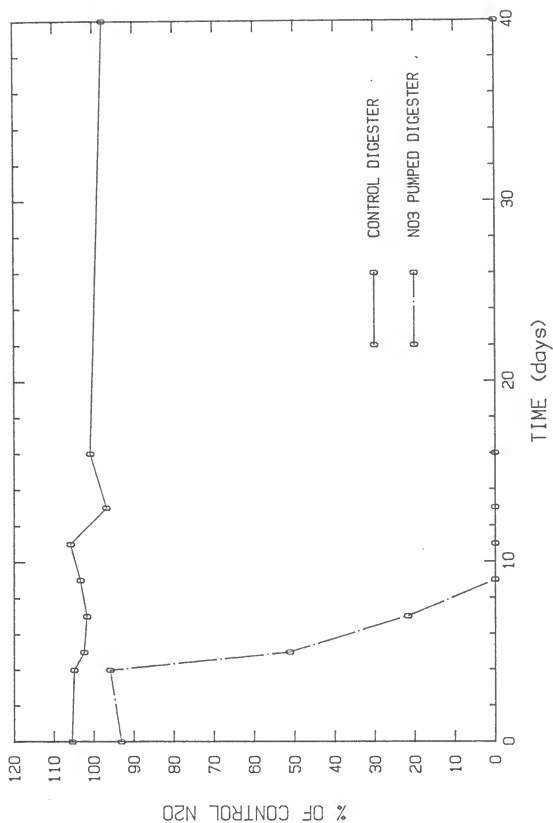


Figure 23. Analysis of recovery of  $^{15}\text{N}$ -labeled nitrate added to nitrate-pumped digester sludge in the ammonium pools produced.

Procedures were as described in Figure 18 except that the 20  $\mu\text{moles NO}_3/\text{ml}$  sludge consisted of 20%  $^{15}\text{N}$ -nitrate (99 atom% purity). Ammonium was determined by the steam distillation method as described in Materials and Methods.  $^{15}\text{N}$  recovery is expressed as a percentage of the  $^{15}\text{N}$ -nitrate added.

% CONTROL GAS RATE or %  $^{15}\text{N}$ -LABEL ADDED

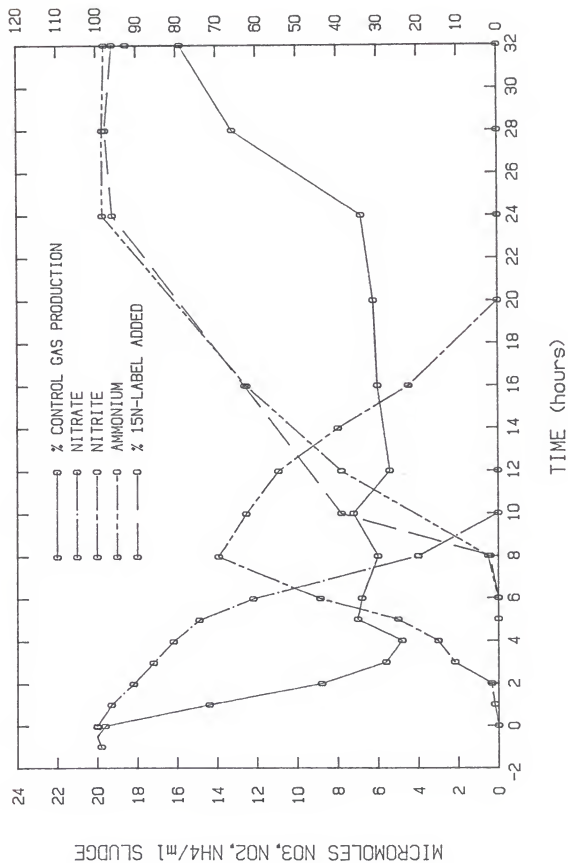
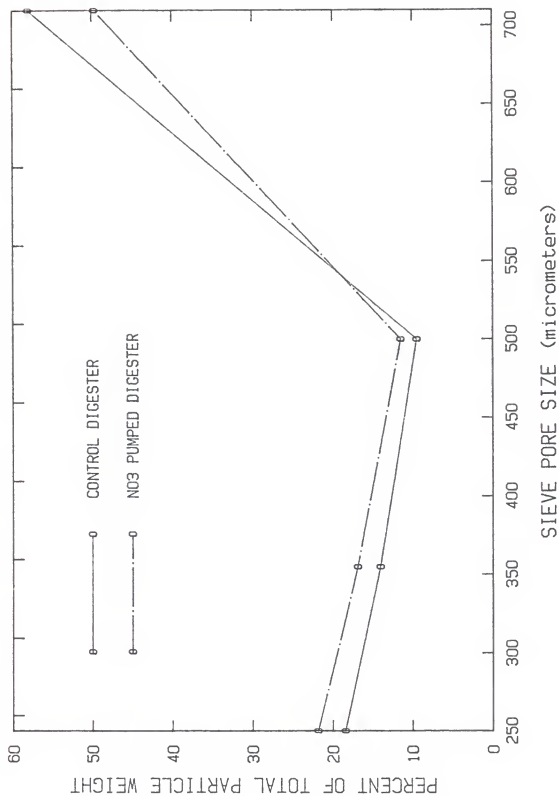


Figure 24. Analysis of particle size distribution in the effluent from control and nitrate-pumped digesters.

Sludge was collected daily, 24 hours after feeding, to cumulative volumes of 400-1000 ml. Particle size distributions were determined as described in Materials and Methods. Particle size distributions were determined from effluent collected from three separate occasions approximately 3 weeks apart. Data represent averages of the three determinations. Standard deviations were within 3%.



## DISCUSSION

The reduction of nitrate to ammonium ion involves enzymatic systems which use reduced pyridine nucleotides as electron acceptors (12). Electrons are used to reduce nitrate and nitrite that would otherwise be used to reduce protons to  $H_2$ . Thus reduction of added nitrate represents a shift in the flow of electrons in the degradation of complex organics in the thermophilic anaerobic digestion system.

### Nitrate Introduction

The digester to which nitrate was added adapted rapidly to nitrate additions (Figures 1,2). Both gas production and acetate pools became less disturbed upon subsequent nitrate additions. A continuous nitrate application of  $0.4 \mu\text{moles NO}_3/\text{ml sludge/hour}$  could be established without digester failure. Increasing the nitrate pumping rate to  $0.5 \mu\text{moles NO}_3/\text{ml sludge/hour}$  was observed to lead to impending digester failure. Digester recovery was nearly complete (essentially 100% of control digester gas production) after only a few days after the nitrate input was shut off. A continuous nitrate pump rate of  $0.4 \mu\text{moles NO}_3/\text{ml sludge/hour}$  was maintained (Figures 3,4). At that nitrate pumping rate, the gas production was 70% of the control's, and acetate pools plateaued at  $58 \mu\text{moles acetate/ml sludge}$ . Ammonium ion pools rose to  $195 \mu\text{moles NH}_4/\text{ml sludge}$  and were maintained.

Ammonium pools in the nitrate-pumped digester sludge increased in proportion to the amount of nitrate added (Figure 5). An analysis

of gas produced from control and nitrate-pumped digesters indicated no differences in nitrogen content (1-2% of gas). Nitrous oxide was not detected in either digester (detection limit: 0.01%). Thus denitrification was determined not to be involved in the reduction of nitrate.

Some parameters on which digester performance are based were not constant over the 24 hour interim between batch feedings.

#### Gas Production and Composition

Cumulative gas production analyzed 24 hours after feeding indicated that the nitrate-pumped digester produced 70% of the control gas (Figure 6), although, upon analysis of relative gas production rates during that 24 hour period, it should be noted that the inhibition varied significantly (60-80%; Figure 7). Thus gas production measurements demand analysis at constant intervals to avoid misinterpretation of digester performance. Gas composition also fluctuated during this time (Figure 8). The highest gas production rates (2-4 hours after feeding) coincided with increased carbon dioxide composition in the gas evolved. Methane comprised a smaller percentage of the gas produced in the nitrate-pumped digester. Decreased methane production (64% of the control) may be attributable in part to decreased methane production from hydrogen and carbon dioxide. The electrons used in the reduction of nitrate were therefore not available for  $H_2$  production. With a decreased supply of substrate ( $H_2$ ), it would be expected that hydrogen-oxidizing methanogens as well as hydrogen-oxidizing sulfate reducers would be lower in numbers in the nitrate-added sludge than in the control sludge. This was the case, as shown in Table 3.

### Changes in Digester Parameters Measured

#### Fatty Acid Pools

Acetate pools underwent changes between feedings (Figure 9). Acetate accumulated faster and was metabolized faster in the control digester sludge than in the nitrate-pumped digester sludge. This was attributed to reduced numbers of acetate-metabolizing methanogens observed in nitrate-pumped digester sludge as compared to those in the control sludge (Table 3).

#### Ammonium Pools

Ammonium pools appeared constant over the 24 hour period after feeding (Figure 10). Table 1 details the ammonium pools in digesters pumped calcium nitrate, sodium nitrate, and ammonium chloride, together with the control digester. As previously discussed, a pumping rate of  $0.4 \mu\text{moles NO}_3/\text{ml sludge/hour}$  results in a buildup of  $196 \mu\text{moles NH}_4/\text{ml sludge}$  if it is assumed that 100% of the nitrate is reduced to ammonium. Indeed, in all three digesters, whether pumping the sodium or the calcium salt of nitrate or pumping ammonium chloride, the ammonium pools reached and remained constant at approximately  $195 \mu\text{moles NH}_4/\text{ml sludge}$ .

#### Sludge pH

Sludge pH remained relatively constant between batch feedings except for a slight decrease (0.1-0.2 pH units) 2-4 hours after feeding (Figure 11). This pH drop coincides with maximum gas production rates and carbon dioxide composition of the gas. The nitrate-pumped digesters exhibited a higher pH than did either the control or the

ammonium chloride-pumped digesters (Table 1). The elevation in pH of the calcium nitrate-pumped digester sludge (7.6) resulted from its higher ammonia concentration and calcium carbonate formed from the calcium ion in the pump solution and carbon dioxide produced by the sludge. In the sodium nitrate-pumped digester, the pH was even higher, probably due to the higher solubility of sodium carbonate compared with its calcium salt.

#### Analysis of Digesters Pumped Calcium Nitrate, Sodium Nitrate, or Ammonium Chloride

An analysis of gas production in calcium nitrate-, sodium nitrate-, and ammonium chloride-pumped digesters indicated the ammonium chloride-pumped digester to be the least inhibited. The ammonium chloride-pumped digester resembled the control digester in volatile fatty acid pools and was similar in pH. Gas production demonstrated less than 8% inhibition. This inhibition may have resulted from the high ammonium or chloride levels. The sodium nitrate-pumped digester exhibited the greatest inhibition. Higher volatile fatty acids and a lower gas production may be attributed to either the high sodium concentration or the high pH.

#### Inhibitory Effects of Nitrate, Nitrite, and Ammonium

The inhibitory effects of nitrate, nitrite, and ammonium were investigated with control and nitrate-pumped digester sludge. Nitrate was more inhibitory to control sludge than to nitrate-pumped digester sludge (Figures 12,15). In both cases, the inhibition of gas production appeared to occur in two stages, with the second stage of

inhibition being more severe and lasting longer. Figures 18 and 19 show that nitrite is an intermediate in the reduction of nitrate to ammonium. The secondary inhibition observed upon nitrate addition is believed to be the result of nitrite levels during accumulation and subsequent reduction. Nitrite was determined to be more inhibitory to nitrate-pumped digester sludge than to control sludge (Figures 13,16), although in both cases it was determined that nitrite was more inhibitory than nitrate when added in equivalent concentrations. Inhibition was single phase and resembled the second phase inhibition found in nitrate inhibition in that a lag was observed before sludge gas production recovery. In both the control and nitrate-pumped digester sludge, added ammonium (up to 20  $\mu\text{moles NH}_4/\text{ml}$  sludge) did not cause inhibition of gas production (Figures 14,17).

#### Reduction of Nitrate and Nitrite

In control sludge, when nitrate was added (20  $\mu\text{moles NO}_3/\text{ml}$  sludge, its reduction began after a lag of 2 hours (Figure 18), whereas in the nitrate-pumped digester sludge, nitrate was reduced without a lag (Figure 19). The lag in nitrate reduction exhibited by the control digester sludge indicated that, although the nitrate reducers were present, their enzymatic systems were not induced. Induction was not warranted because there was no detectable nitrate input to the digester (less than 0.01  $\mu\text{mole NO}_3/\text{ml}$  feed). Nitrate-reducing microorganisms were determined to be in higher numbers in the nitrate-pumped digester sludge than in the control sludge, attributable to the selective nature of continuous nitrate addition to the sludge (Table 3).

Nitrite is reported to be an intermediate in the reduction of nitrate to ammonium ion (17,20,27,39). Consistent with the dissimilatory reduction of nitrate to ammonium, nitrite levels were observed to increase upon nitrate reduction, with subsequent reduction of accumulated nitrite resulting in increases in ammonium pools (Figure 23). In the nitrate addition experiments, accumulated nitrite constituted a greater percentage of the added nitrate in the nitrate-pumped digester sludge than in the control sludge (66 versus 33%; Figures 18, 19). The greater accumulation of nitrite in the nitrate-pumped digester sludge was partially attributed to the slower rate of nitrite reduction in the sludge (Figure 20). Although the nitrate-pumped digester sludge initially reduced nitrite slightly faster than the control digester sludge, an apparent saturation occurred. This was also observed in the ammonium chloride digester sludge in which, after 4 hours, further nitrite reduction was slowed. The high ammonium pools (nitrite reduction end product) in both nitrate- and ammonium-pumped digester sludge may have caused the nitrite reduction reaction to be less favorable below a minimum nitrite concentration.

#### Nitrous Oxide Reduction

Although nitrous oxide was not detected, nor nitrogen composition in the gas phase above levels in the control, the ability of the sludge to carry out denitrification was determined (Figure 22). The control sludge failed to reduce the added nitrous oxide even after 40 days of incubation, whereas the nitrate-pumped digester sludge began reduction after 4 days. The long lag period before the onset of reduction indicates that, although the microorganisms were present,

their enzymatic systems were not normally induced to carry out denitrification.

### Stable Isotope Experiments

To establish more rigorously that essentially all nitrate added to the thermophilic anaerobic digestion system was reduced to ammonium,  $^{15}\text{N}$ -labeled nitrate was added to nitrate-pumped digester sludge (Figure 23). Essentially, all (97.8%) of the label was recovered in the ammonium fraction. The possible  $^{15}\text{N}$ -ammonium incorporated into organic nitrogen was not analyzed. The balance may have been incorporated into cellular material and thus removed from the ammonium pools.

### Analysis of Effluent Composition, Particle Size Distribution, and Microflora

#### Effluent Composition

The control and nitrate-pumped digester sludge were compared with respect to effluent composition (Table 2). The higher percentages of dry matter and volatile solids were the result of increases in volatile fatty acid pools and carbonate levels in the nitrate-pumped digester sludge. The percentages of acid detergent fiber, lignin, and cellulose + cutin were lower in nitrate-pumped digester sludge. Lignin analysis, although lower in the nitrate-pumped digester sludge, was not considered significant. The cutin composition in forages (such as the bermuda grass feed) is extremely low (14); thus decreases in cellulose + cutin reflected increases in cellulose degradation. Increased degradation of cellulose coincided with higher numbers of cellulose-degrading microorganisms in the sludge (Table 3).

### Effluent Particle Size Distribution

An analysis of particle size distribution in the effluent from control and nitrate-pumped digesters revealed that the nitrate addition effected a 10% reduction in the effluent particle sizes. This size reduction may aid in the size reduction of this and other biomass feeds, resulting in increased digestibility.

### Enumerations of Sludge Microflora

Enumerations of cellulose-degrading microorganisms indicate increased numbers in the nitrate-pumped digester sludge as compared to the control digester sludge (Table 3). The enumeration results are consistent with the increased degradation of cellulose in the nitrate-pumped digester sludge (Table 2). It is believed that lower levels of reduced nicotinamide adenine dinucleotide (NADH), resulting from reduction of added nitrate, made the hydrolysis of cellulose and fermentation of resulting sugars thermodynamically more favorable. Decreased levels of NADH would also allow the cellulose to be hydrolyzed more completely.

Nitrate-reducing bacteria were determined to be present in both control and nitrate-pumped digester sludge. Numbers of nitrate-reducing bacteria were higher in the nitrate-pumped digester sludge. The input of nitrate to the control digester was determined to be less than  $0.01 \mu\text{mole NO}_3/\text{ml feed}$ . Without nitrate addition to the sludge, the nitrate-reducing bacteria must carry out alternate energy-yielding reactions, such as the fermentation of sugars. When nitrate is continuously pumped into the sludge, as in the nitrate-pumped digester,

the numbers of nitrate-reducing bacteria are anticipated to increase as the result of the supply of reducible substrate.

The numbers of hydrogen-oxidizing/carbon dioxide-reducing methanogens were reduced 79 fold in the nitrate-pumped digester sludge as compared to the control digester sludge (Table 3). At the same time, the total methane production decreased only 36%. The reduced numbers of hydrogen-metabolizing methanogens in nitrate-pumped digester sludge may be partially the result of either the competition for a reduced supply of substrate ( $H_2$ ) or the toxicity of the added nitrate.

It has been reported in anaerobic sediments and sludge digestion systems that 30% or less of the methane that is produced from the degradation of complex organic matter is derived from  $H_2/CO_2$ . The rest is derived from acetate. The shift in electrons produced in the fermentation system from  $H_2$  production to nitrate reduction would decrease the  $H_2$  substrate supply. Sulfate reducers are reported to have a greater affinity for  $H_2$  than do methanogens (32). The reduced supply of  $H_2$  would therefore be expected to have a greater impact on methane production from  $H_2/CO_2$  than on sulfide production from  $H_2/SO_4$ . It is postulated that this competition for a reduced substrate supply ( $H_2$ ) resulted in small decreases in numbers of sulfate reducers and large decreases in  $H_2$ -oxidizing methanogens. The  $H_2/CO_2$  methanogens have also been reported to metabolize  $H_2$  at a few percent of their potential  $H_2/CO_2$  metabolic rate in sewage sludge (34). If the  $H_2/CO_2$  methanogen numbers were reduced because of the unknown toxic effects of the added nitrate, those cells which remain viable would have the ability to increase significantly their rate of  $H_2/CO_2$  conversion per cell (34).

Acetate-metabolizing methanogens were determined to be reduced in numbers 25 fold in nitrate-pumped digester sludge as compared to control digester sludge. It is proposed that the continuous addition of nitrate was toxic to the cells, resulting in lower numbers. Along with lower numbers of acetate-metabolizing methanogens, the acetate pools in the sludge increased (Table 1). Increases in the metabolic rate of methane production from acetate per cell may account for the discrepancy between total methane production and viable cell numbers.

## CONCLUSION

It has been established that a thermophilic anaerobic digester, batch fed daily, with a 20 day detention time, can be continuously pumped nitrate without digester failure and resulting in a constant inhibition.

The added nitrate has been proven to be reduced exclusively to ammonium through the intermediate nitrate.

The effects of continuous nitrate addition to the thermophilic anaerobic digestion system are an increase in the degradation of cellulose, a decreased total gas and methane production, increased pools of volatile fatty acids and ammonium, and an increased sludge pH.

Although nitrate exhibits its inhibitory effects on sludge more rapidly than does nitrite, nitrite exerts a greater inhibition at equal concentrations, and recovery of gas production occurs after a longer lag period than with nitrate. Ammonium was not found to be inhibitory.

Thus from a practical view, the use of nitrate as an oxidant to increase degradation of complex organic matter in a one stage digestion system is not desirable because of its inhibitory effects on methanogenesis. However, in a multistage digestion system, the addition of nitrate to increase the rate and extent of degradation of complex organic matter may be advantageous. A multistage digestion system may include a primary digestion stage in which the degradation of complex organic matter and conversion to volatile fatty acids are

desired. The inhibition of methanogenesis by nitrate would be an advantage by conserving acetate for the second stage. The particle reduction of the feedstock resulting from nitrate addition would act to increase the feedstock's degradability. The second stage, fed the effluent from the primary stage, would be primarily concerned with the optimum production of methane from volatile fatty acids. The nitrate, reduced to ammonium in the primary stage, would not act to inhibit methanogenesis in the second stage. Effluent from the second stage containing high ammonium levels may be recycled to nitrate through an oxidation stage.

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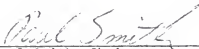
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## BIOGRAPHICAL SKETCH

Christopher John Rivard, son of John F. and Jeanette Y. Rivard, was born December 23, 1956, in Pontiac, Michigan. He graduated from Largo Senior High School, Largo, Florida, in January 1975. He received his Associate of Arts degree from St. Petersburg Junior College, Clearwater, Florida, in June 1976. He attended the University of Florida and earned his Bachelor of Science degree with a major in microbiology and cell science in June 1978. He entered the graduate program in the Department of Microbiology and Cell Science, and in 1981 received a Master of Science degree. He continued his studies at the University of Florida in the Department of Microbiology and Cell Science in pursuit of the Doctor of Philosophy degree.

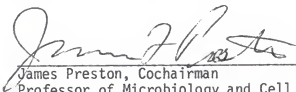
Christoper Rivard is married to Mary Joann (Cochrane) Rivard. They have three children, John Christopher, age 5, Sean Sebastian, age 3, and Beth Ann, age 4 months.

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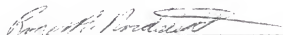
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December 1983



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